

GROWTH FACTORS IN INVERTEBRATE IN VITRO CULTURE

STEPHEN M. FERKOVICH AND HERBERT LOBERLANDER

*Insect Attractants, Behavior, and Basic Biology Research Laboratory, Agricultural Research Service,
U.S. Department of Agriculture, Gainesville, Florida 32604*

(Received 3 January 1991; accepted 8 February 1991)

SUMMARY

An increasing number of polypeptide growth factors have been identified that have proven essential in the development of defined cell culture media for mammalian cell culture. The development of defined mammalian cell culture media, in turn, has provided an environment for studying cell lines in an experimentally manageable unit for studying the action of cellular regulators and genes that determine the properties of cells. Evidence that vertebrate growth factors may be present in insects is based on DNA sequences that encode epidermal growth factor and transforming growth factor- β . However, research on the influence of commercially available vertebrate growth factors is very limited. Although the majority of insect growth-promoting substances studied were isolated directly from insect hemolymph, few of these have been purified to the extent that they could be tested in insect cell, tissue, and endoparasite cultures. Research is needed in both of these areas to aid in developing defined insect culture systems, and to understand better the regulation of postembryonic growth and development in insects.

Key words: growth factor; invertebrate; insect; cell line; polypeptide; hemolymph.

INTRODUCTION

The importance of growth factors in regulating the proliferation of vertebrate cells has been recognized for many years (14). In the first issue of the new journal *Progress in Growth Factor Research* (1989, Vol. 1), Dr. John Heath, the Executive Editor, defined growth factors as a "... diverse group of polypeptide regulatory agents which act to control a host of cellular responses (not least cell multiplication) by mechanisms analogous to classical endocrine hormones" (18). Increased interest in these agents has occurred because of the discovery that viral oncogenes encoded growth factors and their receptors and that some genes are activated by polypeptide growth factors (29). Vertebrate growth factors are a diverse group of agents. Many of them have been characterized and their mode of action has been defined, for reviews see Gospodarowicz and Moran (14), Barnes and Sirbasku (3), and Deul (8), and for their role in the development of defined cell culture systems see McKeehan et al. (29). Briefly, growth factors range in molecular mass from 6000 Da for the epidermal growth factor to 80 000 Da for the iron-transporting factor, transferrin. Some of them act externally through cell surface receptors such as epidermal, platelet-derived, and insulinlike factors, whereas some have binding proteins associated with them. Insulinlike growth factors, such as IGF-1 and IGF-2, have different binding proteins that can modulate the actions of the factors in both inhibitory and stimulatory ways (4). In addition, there are growth-factor-specific enzymes that may be important in regulating levels of the growth factor. An example is the evolutionarily conserved insulin-degrading enzyme that degrades the alpha-transforming growth factor after it acts on the target cell (12).

Although a major goal of insect cell culture specialists is to concoct new and better growth media for culturing cells and tissues, little effort has been directed toward isolating and identifying growth factors in invertebrates. A review of growth factors in invertebrates revealed that much less is known about their production, structure, and mode of action than about any of the well-characterized vertebrate growth factors (Table 1) (1,5,9-11,16,19,21,24,25,33,35-39). Molecular size of most of the insect growth factors was determined primarily by size exclusion during dialysis after very limited purification. Most of these factors are low molecular weight compounds (<10 kDa) that have not yet been purified to the point of providing molecular identity, such as the cationic factor (39), and the *Bombyx* growth-promoting substances (1,35). Except for the cationic growth factor (39), the source for all of the reported insect growth factors was hemolymph. Some of the growth factors studied had apparent molecular weights greater than 10 000. For example, a heat labile (>10 kDa) substance was found in silkworm pupae that stimulated maturation of spermatocytes in vitro (38). Also, a nondialyzable substance (>10 kDa) in the hemolymph of the silkworm was attributed with stimulating growth in a cell line derived from mosquito larvae (MSQ) (19). Two low molecular weight polypeptides, the vesicle promoting factor (10,11), and the insulinlike factor (9) have been partially sequenced. Two other polypeptides which have been isolated and partially characterized include the largest growth factor identified, a 230 kDa polypeptide that stimulated parasitoid egg development in vitro and was isolated from host hemolymph (16). Also, a nonstorage oligomeric protein isolated from larval and pupal hemolymph of *Manduca sexta* induced intermolt cuticular deposition in vitro (37).

Some of the most convincing evidence for growth factor hetero-

TABLE 1
GROWTH FACTORLIKE SUBSTANCES IN INSECTS

Factor	Source	M.M., Daltons	Characterization	Biological Action	References
Cationic Growth Factor	adult extracts <i>Drosophila melanogaster</i>	<400	? no amino acids present	Proliferation of KC HP cell line	(39)
Pupation factor	<i>Manduca sexta</i>	<1000	? 2 or more molecules (sugar or peptide)	Induces pupation in egg parasitoid	(21)
Growth Promoting Substance	<i>Bombyx mori</i> hemolymph	<5000	neutral lipid fraction	proliferation of cell line	(35)
Growth Promoting Substance	<i>Bombyx mori</i> hemolymph	<10 000 dialyzable	Acid and alkali soluble	culture of tissue	(1)
Growth Promoting Substance	hemolymph	>10 000 nondialyzable	heat labile	spermatogenesis	(38)
Growth Promoting Substance	<i>Antheraea pernyi</i> hemolymph	>10 000 nondialyzable		proliferation MSQ cell line	(19)
Vesicle Promoting Factor	<i>Trichoplusia ni</i> hemolymph	16 900	nonglycosylated polypeptide sequenced	induces formation of vesicles in cell line	(10, 11)
Insulinlike	<i>M. Sexta</i> <i>D. melanogaster</i> <i>C. vomitoris</i> larvae and KC cells	24 400	amino acid and DNA sequenced	regulates carbohydrate metabolism	(9)
Egg Development Stimulating Protein	<i>Heliothis zea</i> hemolymph	230 000	glycosylated polypeptide	promotes egg development of endoparasite	(16)
Hemolymph Trophic Factor	<i>M. Sexta</i> hemolymph	286 000	tetrameric protein	cuticle deposition in vitro	(37)
Epidermal-like growth factor	<i>Drosophila</i>		DNA sequences in notch and delta genes encode EGF-like molecules	determinants of ectodermal cells in embryo	(24, 25, 36)
Transforming-like growth factor	<i>Drosophila</i>		DNA sequences in DPP-C gene complex encode TGF-B	evidence that regulates cell division and differentiation	(5, 33)

phyly is based on studies of gene regulation in *Drosophila* embryogenesis. Considerable DNA sequence similarity exists between the genes that encode the Notch, Delta, and decapentaplegic proteins and the vertebrate growth factors such as the epidermal growth factor (24,25,36) and the transforming growth factor- β (5,33). However, information on most insect growth regulators is limited, and compared with the progress made in the field of vertebrate growth factors the status of growth factors in insects and invertebrates is in its infancy. Currently, insect cell biologists do not have specific growth factors available to them to formulate chemically defined media for insect cells.

If development of tissue culture media for insect cell lines is examined from a historic perspective, we can trace the use of supplemental factors from Grace (15), who established the first insect cell line from *Antheraea*, a silkworm. Later, lobster hemolymph was used for a short period of time (6,32,40), and then a number of cell culturists replaced hemolymph with fetal bovine serum for cell and tissue culture. Fetal bovine serum seems to be the most frequently used vertebrate serum, and according to Mitsuhashi and Goodwin (31), most primary insect cell cultures cannot grow without it. Barnes and Sato (2) successfully used growth factors, hormones, binding proteins, and attachments factors to replace serum in a defined media for vertebrate cell lines, but this has not yet been achieved for insect cell culture media.

Defined cell culture media have been achieved for vertebrate cell lines (2). Because arthropod blood can be replaced with vertebrate

serum, which in turn can be replaced by growth factors in vertebrate cell lines, we asked whether vertebrate serum could be replaced by vertebrate growth factors for use with insect cell lines. However, published information on the effect of vertebrate growth factors on insect cell lines is very limited (30). Attempts have been made to isolate growth factors that may affect insect cells from non-insect sources such as fetal bovine serum (26). The nondialyzable portion of fetal bovine serum stimulated the growth and development of embryos and larvae of a parasitic wasp (17), and Mitsuhashi (30) found that fetal bovine serum contained heat and pronase-sensitive substances that promoted growth of an insect cell line. In addition to growth factors in fetal bovine serum, insulin functioned as an insect growth factor because it promoted growth of a *Drosophila melanogaster* cell line and imaginal discs, although not of lepidopteran cell lines (7,34). Lynn and Hung (28) successfully employed Ultraser G and Nuserum, two serum substitutes, to supply mammalian growth factors required for growth of a continuous cell line from an insect egg parasitoid. However, the investigators were unable to culture the cells using purified growth factors as a substitute for the serum.

We are aware of two additional published reports on the effect of vertebrate growth factors on insect cell lines. Glycylhistidyllysine, a vertebrate copper-transporting growth factor, stimulated cell growth in serum-free media for Lepidopteran cell lines (13). Epidermal growth factor and fibroblast growth factor were beneficial in culturing differentiated and venom gland cells (28). We also tested the effects of selected vertebrate growth factors on an insect cell line

TABLE 2

EFFECTS OF VERTEBRATE GROWTH FACTORS
ON INSECT CELL GROWTH

Medium Graces TC, % FBS	IAL-PID ₂ Cell Line Factors	Growth, %
10	—	100
5	—	54
5	FGF	42
5	EGF	53
5	FGF + EGF	53
5	FGF, EGF + ITS	46
(Concentrations)		
FBS = fetal bovine serum	10/5%	
FGF = fibroblast growth factor	25 ng/ml	
EGF = epidermal growth factor	25 ng/ml	
ITS = insulin; transferrin; selenium	62.5 µg; 62.5 µg; 62.5 ng/ml	

(IAL-PID₂) (27) derived from imaginal wing discs of the Indian-meal moth (Table 2). Reducing the concentration of fetal bovine serum by 50% reduced growth of the cells by 50%. However, the addition of fibroblast growth factor, epidermal growth factor alone or in combination with insulin, transferrin, or selenium did not stimulate growth of the cells. These results suggest that specific peptides or growth factors may substitute for the fetal bovine serum in some insect cultures.

Insect hemolymph has been a source of factors with potential for use in tissue culture. For example, two iron-binding proteins have been identified in the hemolymph of *Manduca sexta*: a high molecular weight protein (490 kDa) functionally comparable to ferritin in mammals, and an 80 kDa protein comparable to transferrin (20). These iron-binding proteins are thought to allow lepidopterans to store large amounts of iron during the larval feeding stage and to mobilize it during the development of flight muscle in the adult. Purified proteins such as these could be tested on lepidopteran cell lines. Other proteins such as storage proteins, transport proteins, and bactericidal proteins have been identified from *in vitro* studies and have critical functions attributed to them (reviewed in 22), these proteins might also be beneficial for the growth of insect cell lines.

Thus, it is not yet certain that there are polypeptide growth factors present in nonendocrine insect tissues that are involved in cellular activities *in vivo*. Although vertebrate growth factors have not routinely served as growth factors for insect cells, there still may be specific growth factors in the insect's hemolymph that would aid in formulating chemically defined media for insect cells. In *Drosophila*, as mentioned above, there is evidence that there are vertebrate-like growth factors with similarities to epidermal growth factor and transforming growth factor molecules that function during embryogenesis (1,5,9–11,16,19,21,24,25,33,35–39).

Perhaps the reason there is such a lack of information on invertebrate growth factors is that they are difficult to isolate and identify, even with advances in high performance purification and analytical methods (14). They occur in low concentrations in tissues among the multiplicity of other bioactive components present. When the materials are tested, the cells must be maintained under conditions such that only putative growth factors are limiting, to allow the effect of a candidate growth factor to be evident upon addition. The bioassay also must be specific. For example, if the cells respond to two

different growth factors initiating DNA synthesis, a bioassay based on the amount of DNA synthesis induced would not distinguish between the two factors. Additional difficulties could be co-purification of an inhibitor with the growth factor, or further production of growth factors by the cells which would interfere with the bioassay. In addition, binding proteins could be involved which can further complicate the purification. For example, the insulinlike growth factors in human plasma have binding proteins that are recognized as modulators of the factor's activity. IGF activity was observed to exist in plasma at a molecular mass above 50 kDa by gel permeation chromatography. By lowering the pH, activity can be converted to a lower molecular weight, that of the free IGF (23). These problems are not necessarily greater for insects than for vertebrates, and we believe too little effort has been expended in this area.

Perspectives

As a first step, a systematic study is needed on the effects of commercially available vertebrate growth factors on selected insect cell lines and tissues. Then, greater emphasis needs to be given to specific cell and organ culture bioassays to discern whether invertebrate growth and morphogenic factors exist. Research is needed on the structural characterization and genetic control of these growth factors in invertebrates. Once the growth factors are characterized they could be produced, and such availability of insect growth factors would aid in formulating chemically defined media, as has been done for various vertebrate cell lines. Moreover, this approach will help uncover insect-specific cellular regulators and genes controlling the proliferation and differentiation of insect cells, tissues, and embryos.

REFERENCES

1. Aizawa, K.; Sato, F. Culture de tissus de ver a soie, *Bombyx mori*, dans un milieu sans hemolymph. Ann. Epiphyt. 14:125; 1963.
2. Barnes, D.; Sato, G. Serum-free cell culture: a unifying approach. Cell 22:649–655; 1980.
3. Barnes, D.; Sirbasku, editors. Peptide growth factors, Methods in Enzymology, vols. 146,147. New York: Academic Press; 1987.
4. Baxter, R. C.; Martin, J. L. Binding proteins for the insulin-like growth factors: structure, regulation and function. Prog. Growth Factor Res. 1:49–68; 1989.
5. Bryant, P. J. Localized cell death caused by mutations in a *Drosophila* gene coding for a transforming growth factor- β homolog. Dev. Biol. 128:386–395; 1988.
6. Chiu, R.-J.; Black, L. M. Monolayer cultures of insect cell lines and their inoculation with a plant virus. Nature 215:1076–1078; 1967.
7. Davis, K. T.; Shearn, A. In vitro growth of imaginal disks from *Drosophila melanogaster*. Science 196:438–440; 1977.
8. Deul, T. F. Polypeptide growth factors: roles in normal and abnormal cell growth. Ann. Rev. Cell Biol. 3:443–492; 1987.
9. Ebberink, R. H. M.; Smit, A. B.; Van Minnen, J. The insulin family: evolution of structure and function in vertebrates and invertebrates. Biol. Bull. 177:176–182; 1989.
10. Ferkovich, S. M.; Oberlander, H.; Dillard, C., et al. Purification and properties of a factor from insect hemolymph that promotes multicellular vesicle formation *in vitro*. Arch. Insect Biochem. Physiol. 6:73–83; 1987.
11. Ferkovich, S. M.; Oberlander, H.; Dillard, C., et al. Identification of a cell line vesicle promoting factor in larval tissues of *Trichoplusia ni*. In: Hagedorn, H. H.; Hildebrand, J. G.; Kidwell, M. G., et al., eds. Molecular insect science. New York and London: Plenum Press; 1990:301.
12. Garcia, J. V.; Gehm, B. D.; Rosner, M. R. An evolutionarily conserved

- enzyme degrades transforming growth factor- α as well as insulin. *J. Cell Biol.* 109:1301–1307; 1989.
13. Goodwin, R. H.; Adams, J. R. Nutrient factors influencing viral replication in serum-free insect cell line culture. In: Kurstack, E.; Maramorosh, K.; Dubendorfer, A., eds. *Invertebr. systems in vitro*. 16:493–509; 1980.
 14. Gospodarowicz, D.; Moran, J. S. Growth factors in mammalian cell culture. *Ann. Rev. Biochem.* 45:531–558; 1976.
 15. Grace, T. D. C. Establishment of four strains of cells from insect tissue grown *in vitro*. *Nature* 195:788–789; 1962.
 16. Greany, P.; Clark, W.; Ferkovich, S. M., et al. Isolation and characterization of a host hemolymph protein required for development of the eggs of the endoparasite *Microplitis croceipes*. In: Hagedorn, H. H.; Hildebrand, J. G.; Kidwell, M. G., et al., eds. *Molecular insect science*. New York and London: Plenum Press; 1990:306.
 17. Greany, P. D.; Ferkovich, S. M.; Hanrahan, A. M. Use of dialyzed, concentrated fetal bovine serum as a medium supplement for the endoparasitoid *Apanteles marginiventris* (Cresson). VI International Conference on Invertebrate Tissue Culture. Abstracts, E. Kurstack, University of Montreal, Canada. 1983:78.
 18. Heath, J. K. Progress in growth factor research editorial. *Prog. Growth Factor Res.* 1:i–ii; 1989.
 19. Hsu, S. H.; Liu, H. H.; Sutor, E. C., Jr. Further description of a subline of Grace's mosquito (*Aedes aegypti* L.) cells adapted to hemolymph-free medium. *Mosq. News* 29:439–446; 1969.
 20. Hubners, H. A.; Hubners, E.; Webb, B. A., et al. Iron binding proteins and their roles in the tobacco hornworm, *Manduca sexta* (L.). *J. Comp. Physiol.* B158:291–300; 1988.
 21. Irie, K.; Xie, Z.; Nettles, W. C., Jr., et al. The partial purification of a Trichogramma pretiosum pupation factor from hemolymph of *Manduca sexta*. *Insect Biochem.* 17:269–275; 1987.
 22. Kanost, M. R.; Kawooya, J. K.; Law, J. H., et al. Insect haemolymph proteins. *Adv. Insect Physiol.* 22:299–396; 1990.
 23. Kaufmann, U.; Zapf, J.; Torretti, B., et al. Demonstration of a specific carrier protein of nonsuppressible insulin-like activity *in vivo*. *J. Clin. Endocrinol. Metab.* 44:160–166; 1977.
 24. Kelley, M. R.; Kidd, S.; Deutsch, W. A., et al. Mutations altering the structure of epidermal growth factor-like coding sequences at the *Drosophila* notch locus. *Cell* 51:539–548; 1987.
 25. Kopczynski, C. C.; Alton, A. K.; Fechtel, K., et al. Delta, *Drosophila* neurogenic gene, is transcriptionally complex and encodes a protein related to blood coagulation factors and epidermal growth factor of vertebrates. *Genes Dev.* 2:1723–1735; 1988.
 26. Kuno, G.; Hink, W. F.; Briggs, J. D.; Growth-promoting serum proteins for *Aedes aegypti* cells cultured *in vitro*. *J. Insect Physiol.* 17:1865–1879; 1971.
 27. Lynn, D. E.; Oberlander, H. Obtainment of hormonally sensitive cell lines from imaginal discs of Lepidoptera species. In: *Techniques in in vitro invertebrate hormones and genes. Techniques in the life sciences; cell biology*, vol. C2. Ireland: Elsevier Scientific Publishers; 1986:1–12.
 28. Lynn, D. E.; Hung, A. C. F. Development of a continuous cell line from the insect egg parasitoid, *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae). *In Vitro Cell. Dev. Biol.* 22:440–442, 1986.
 29. McKeehan, W. L.; Barnes, D.; Reid, L., et al. Frontiers in mammalian cell culture. *In Vitro Cell. Dev. Biol.* 26:9–23; 1990.
 30. Mitsuhashi, J. Nutritional requirements of insect cell *in vitro*. In: Mitsuhashi, J., ed. *Invertebrate cell system applications*. Boca Raton, FL: CRC Press; 1989:3–20.
 31. Mitsuhashi, J.; Goodwin, R. H. The serum-free culture of insect cells *in vitro*. In: Mitsuhashi, J., ed. *Invertebrate cell system applications*. Boca Raton, FL: CRC Press; 1989:31–43.
 32. Oberlander, H. Growth and partial metamorphosis of imaginal disks of the greater wax moth, *Galleria mellonella*, *In vitro*. *Nature* 216:1140–1141; 1967.
 33. Padgett, R. W.; St. Johnson, R. D.; Gelbart, W. M. A transcript from a *Drosophila* pattern gene predicts a protein homologous to the transforming growth factor-B family. *Nature* 325:81–84; 1987.
 34. Seecof, R. L.; Dewhurst, S. Insulin is a *Drosophila* hormone and acts to enhance the differentiation of embryonic *Drosophila* cells. *Cell Differ.* 3:63–70; 1974.
 35. Vaughn, J. L.; Louloudes, S. J. Isolation of two growth promoting fractions from insect hemolymph. *In Vitro* 14:351; 1978.
 36. Wharton, K. A.; Johansen, K. M.; Xu, T., et al. Nucleotide sequence from the neurogenic locus Notch implies a gene product that shares homology with proteins containing EGF-like repeats. *Cell* 43:567–581; 1985.
 37. Wielgus, J. J.; Caldwell, G. A.; Nichols, R. L., et al. Purification, properties, and titer of a hemolymph trophic factor in larvae and pupae of *Manduca sexta*. *Insect Biochem.* 20:65–72; 1990.
 38. Williams, C. M.; Kambyseilis, M. P. *In vitro* action of ecdysone. *Proc. Natl. Acad. Sci. USA* 63:231; 1969.
 39. Wyss, C. CalGF, a cationic low molecular weight growth factor from *Drosophila melanogaster* and the nutritional requirements of kchp cells. *Insect Biochem.* 12:515–522; 1982.
 40. Yunker, C. E.; Vaughn, J. L.; Cory, J. Adaptation of an insect cell line (Grace's Antheria cells) to medium free of insect hemolymph. *Science* 155:1565–1566; 1967.